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Ultra-Low Dispersity Poly(vinyl alcohol) Reveals Significant Dispersity Effects on Ice Recrystallization Inhibition Activity

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ABSTRACT: Polymer mimics of antifreeze proteins are emerging as an exciting class of macromolecular cryoprotectants for the storage of donor cells and tissue. Poly(vinyl alcohol), PVA, is the most potent polymeric ice growth inhibitor known, but its mode of action and the impact of valency (DP) are not fully understood. Herein, tandem RAFT polymerization and column chromatography are used to isolate oligomers with dispersities <1.01 to enable the effect of molecular weight distribution, as well as length, to be probed. It is found that polymers with equal number average molecular weight, but lower dispersity, have significantly less activity, which can lead to false positives when identifying structure-property relationships. The minimum chain length for PVA's unique activity, compared to other non-active poly-ols was identified. These results will guide the design of more active inhibitors, better cryopreservatives and a deeper understanding of synthetic and biological antifreeze macromolecules.

Antifreeze proteins and antifreeze glyco proteins (AF(G)Ps) have evolved the unique function of binding to and inhibiting the growth of ice crystals.^{1–3} The ability to slow ice growth has major technological applications in aerospace, wind farms⁴ and in the cryopreservation of donor cells and tissue, which are traditionally stored using toxic organic solvents.⁵ Synthetic mimics of AF(G)Ps have recently emerged, which have few structural similarities but can reproduce AF(G)P properties.^{6,7} The most desirable property for cryopreservation is ice recrystallization inhibition (IRI) which prevents ice-growth during thawing.⁸ Various IRI active materials and their use in cryopreservation have been reported including poly(vinyl alcohol)^{9–11} poly(ampholytes)^{12,13}, amphipathic metallo-helices¹⁴ and graphene oxide derivatives.¹⁵ Whilst the interactions between antifreeze proteins and ice has led to identification of ice-binding domains^{3,16} how the most active synthetic polymer mimic, PVA, functions remains unclear, but amphipathicity appears to play a role.^{14,15} Inada *et al.*¹⁷ and Gibson and coworkers¹⁸ have demonstrated that PVA's activity increases with increasing molecular weight. Most other poly-ols (and most polymers) have essentially zero IRI activity highlighting that the presence of hydroxyls is not the key motif for activity.^{19,20} Budke and Koop have postulated that a minimum of 10 repeat units are essential for PVA to function, based on lattice matching with ice crystal surfaces.²¹ This is supported by statistical co-polymerizations of PVA (spacing out the hydroxyls) which dramatically reduces IRI activity,¹⁸ but block copolymerization is a tolerated modification.^{22,23} Externally triggerable IRI's have been obtained which use supramolecular self-assembly to increase the DP of PVA from 10 to 30, and hence the activity of PVA 'turns on'.²⁴ However, identification of the minimum chain length for activity is non-trivial due to the inherent dispersity of polymers meaning even those derived from controlled radical polymerization are complex mixtures.

Recent advances in single-monomer insertion,²⁵ and attempts to obtain sequence-regulated synthetic polymers^{26–28} have highlighted the importance of minor sequence modifications on emergent macromolecular properties such as self-assembly²⁹ single chain folding³⁰ and lectin binding.³¹ Stubbs *et al.* have demonstrated that alternating poly(ampholyte) co-polymers have superior ice recrystallization inhibition activity relative to random copolymers.³² Hawker and coworkers have recently introduced the use of silica gel column chromatography to purify low-molecular weight (DP<20) polymers derived from radical polymerization such that near-monodisperse fractions can be easily isolated, dramatically reducing dispersity.³³ Using this method, the self-organization of oligo(methyl methacrylate)-block-oligo(dimethylsiloxane) was found to be markedly different from those with increased dispersity. Considering the above, here we report the synthesis and purification of ultra-low dispersity oligo(vinyl alcohol) and quantitative evaluation of the impact of each fraction on its IRI activity. We show a profound effect of the chain length distribution, with tiny amounts of high molecular weight material, even in 'narrow' polymers derived from RAFT, having dramatic effects on ice interaction.

To obtain well-defined PVA, RAFT/MADIX polymerization³⁴ of vinyl acetate was conducted using methyl (ethoxy carbonylthio) sulfanyl acetate as the chain transfer agent (CTA) and azobis(4-cyanovaleric acid) (ACVA) as the radical source, Figure 1A.¹⁸ RAFTed polymers with average DPs from 6 – 13 were subjected to column chromatography using a procedure from Hawker *et al.*, with monitoring by thin layer chromatography (Figure 2B).³³ Fractions enriched in single oligomers were obtained, resulting in significant narrowing of their dispersity in size exclusion chromatography (ESI). The PVACs were analysed by electrospray ionisation mass spectrometry, enabling molecular weight and distribution to be calculated. From

these, a range were selected with PVAc dispersities < 1.01, covering a range DPs; in terms of the purity of the major peak this is an increase from <10% to >60%. PVA was generated by deacetylation with sodium ethoxide followed by purification with ion-exchange resins. ¹H NMR and infrared spectroscopy (Supp. Info.) confirmed quantitative removal of the acetate groups. Fractionated chain lengths > 15 could not be isolated as the method is only suitable for oligomers.³³

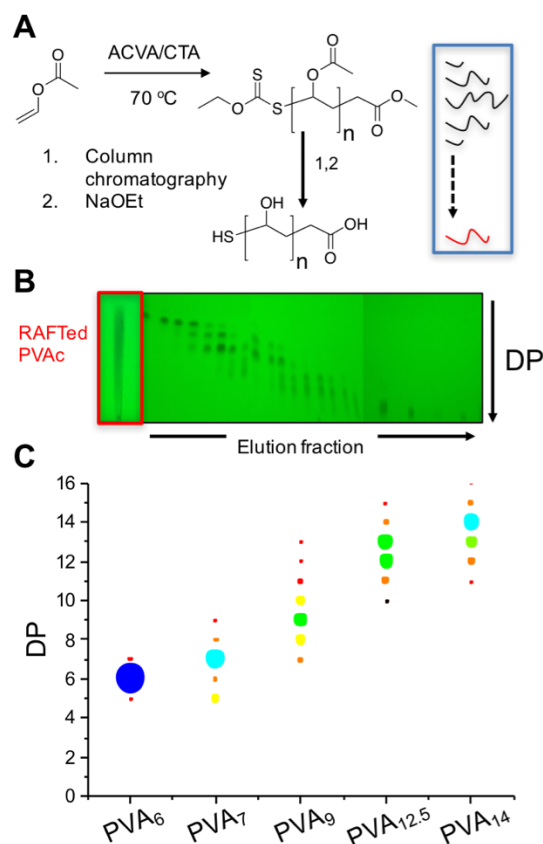


Figure 1. A) Synthesis of PVA by RAFT/MADIX polymerization; B) Thin layer chromatography of columned polymers; C) Chain length distribution frequency of polymers used here.

Table 1. RAFTed and column-purified polymers

Entry	$M_n^{(d)}$ (g.mol ⁻¹)	DP (-)	\bar{D} (-)
RAFT-PVA ₆	540	6	1.03 ^(a)
RAFT-PVA ₁₁	680	11	1.14 ^(a)
RAFT-PVA ₁₃	820	13	1.10 ^(a)
PVA ₆	460	6	1.005 ^(b)
PVA ₇	500	7	1.009 ^(b)
PVA ₉	590	9	1.01 ^(b)
PVA _{12.5}	745	12.5 ^(c)	1.007 ^(b)
PVA ₁₄	810	14	1.006 ^(b)

(a) Data from SEC of PVAc precursors, expressed as PVA equivalent for comparison; (b) Calculated from ESI-MS; (c) DP 12 and 13 were equal as the major peaks; (d) M_n is PVA equivalent not PVAc

A 'splat' assay was used to evaluate the ice recrystallization inhibition (IRI) activity of the oligomers.³⁵ A 10 μ L droplet at each concentration in PBS was dropped onto a chilled (-80 °C) glass

slide, before being transferred to a cold stage set at -8 °C, under N₂. After 30 minutes the wafer was imaged and the mean grain size (MGS) was calculated relative to a PBS control, with smaller numbers indicating more activity. Figure 2A compares the IRI activity of RAFTed PVA₁₁ compared to the low-dispersity polymers at 10 mg.mL⁻¹. As expected the shortest polymers (PVAs 6-9) had low activity with MGS ~ 80 %, identical to a negative control (poly(ethyleneglycol)). However, PVA₁₄ which had a higher number average molecular weight than RAFTed PVA₁₁, had lower activity (50 % MGS versus 20 %). To a first approximation, this is contrary to previous results which show that increased M_n increases activity.^{18,24} However, it provides the first evidence that high molecular weight fractions, even within a RAFTed polymer distribution, dominates the IRI activity. To probe this in more detail, the entire library was evaluated across a full range of concentrations, Figure 2B. At all concentrations, PVA₁₁ with higher dispersity was significantly more active than all other fractions.

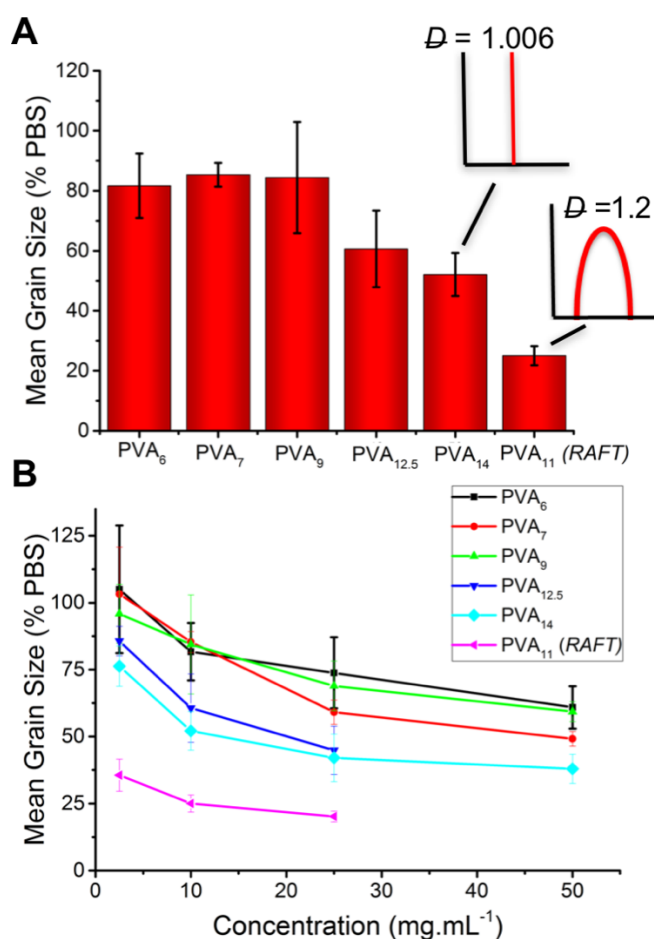


Figure 2. IRI activity of polymers. A) Comparison of IRI activity of purified versus RAFTed PVA at 10 mg.mL⁻¹; B) Variable concentration IRI activity. Error bars represents \pm S.D from minimum of 3 repeats.

The above data shows that small fractions of high molecular weight impurities dominate the observed IRI. For translation to biomedical applications, understanding the role of the entire distribution is essential, particularly to reduce dosage through removal of inactive components. Figure 3 depicts the

molecular weight distribution of the polymers purified here, compared to the starting distribution from a RAFTed polymer. Example ice wafers of two polymers with similar M_n but different dispersity are shown in Figure 3B

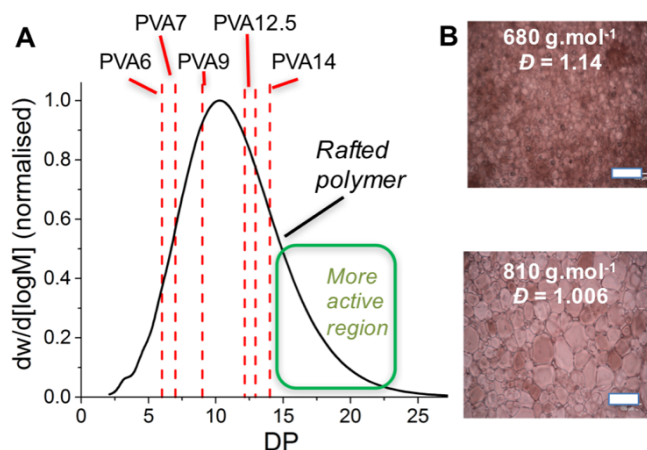


Figure 3. Distribution of PVA11 by RAFT compared to the narrow fractions isolated here. Scale bar is 100 μm .

The above data raises the question of *when does PVA become IRI active* compared to other poly-ols? PVA is unique in its IRI activity compared to other poly-ols.^{19,36} Koop has hypothesized that the hydroxyl spacing in PVA presents a match to the primary and secondary prism faces of ice, and that a minimum of approximately 10 units is required for activity.²¹ To determine the critical IRI chain length, a mono and di-saccharide were used as controls. Saccharides have regularly spaced hydroxyls and hence can be considered as oligomers, to compare against PVA.³⁷ At 50 mg.mL^{-1} glucose (OH = 4), and trehalose (OH = 8) had essentially identical activity to PVA₆, PVA₇ and PVA₉, indicating that at this length there was no specific IRI activity. (It should be noted that these high concentrations any additive will have some weak, non-specific growth inhibition.) However, PVA_{12.5} and PVA₁₄ were significantly more active and maintained activity to lower concentrations (25 mg.mL^{-1}), indicating this is the threshold for activity. It is important to note that these are still vastly less active than e.g. PVA₂₀ which functions at 20 X lower concentrations. The use of ultra-low dispersity polymers to identify the minimum chain length will help to understand the unique activity of PVA and to develop new IRI active macromolecules for translational biomedical applications, as well as enabling a link to modelling/simulation of this complex interface.

In conclusion, we have demonstrated that column chromatography can be used to access ultra-low dispersity PVA's, with dispersity values as low as 1.005. Using these oligomers, the critical chain length for biomimetic ice recrystallization inhibition activity was identified, with PVA showing onset ('switch on') of activity at 12 units. Such insight will help improve our theoretical understanding of this extremely complex interface. It also provides insight into the role of dispersity in emergent applications of polymers; in this case, the high molecular weight tail dominates all observed ice interacting properties and will guide the use of substantially lower therapeutic doses of polymers by removing inactive (or too active) fractions.

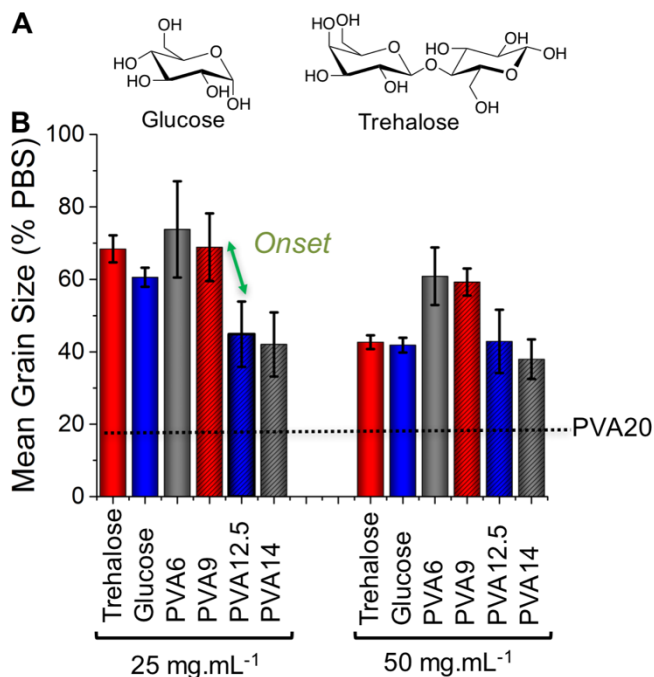


Figure 4. IRI activity of sugars versus PVA. A) sugars used; B) Comparison of IRI activity of saccharides versus PVAs. Error bars represents \pm S.D from minimum of 3 repeats.

ASSOCIATED CONTENT

Experimental procedures and characterization data, including mass spectrometry and size exclusions chromatography analysis is included in the Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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